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Stem cell potency and the ability to contribute to chimeric organisms

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Abstract

Mouse embryonic chimeras are a well-established tool for studying cell lineage commitment and pluripotency. Experimental chimeras were successfully produced by combining of two or more preimplantation embryos or, by introduction into a host embryo cultured pluripotent embryonic stem cells (ESCs). Chimera production using genetically modified ESCs became the method of choice for generation of knockout or knockin mice. Although the derivation of ESCs or ESC-like cells has been reported for other species, only mouse and rat pluripotent stem cells have been shown to contribute to germline competent chimeras, which is the defining feature of ESCs. Herein we describe different approaches employed for the generation of embryonic chimeras, define chimera competent cell types and describe cases of spontaneous chimerism in humans. We also review the current state of derivation of pluripotent stem cells in several species and discuss outcomes of various chimera studies when such cells are used.

Developmental potential and embryonic chimerism

The term chimerism is derived from the Greek Χίμαιρα ‘she-goat or monster’, a monstrous fire-breathing creature composed of the parts of three animals: a lion, a serpent and a goat (<http://en.wikipedia.org/wiki>). In biology, the term chimera usually refers to a single organism composed of two or more different populations of genetically distinct cells originated from different zygotes. Typically, experimental embryonic chimeras are formed by aggregation of two or more whole early cleaving embryos or by combining isolated blastomeres from two or more embryos. The contribution level of the each parental cell types in tissues and organs of chimeric offspring can vary. For example, a chimeric organism could potentially consist of an equal mixture of parental embryonic cells in all cell and tissue types or contain only limited contribution of one of the genotypes in some tissues (microchimerism or Mc).

The level of contribution in embryonic chimeras is largely depends on the developmental potency of parental cells. Mammalian development originates from a state of totipotency, an attribute of zygotes and early cleaving blastomeres. Totipotency is defined as the ability of a single cell to divide and produce all the differentiated cells of an organism including extra-embryonic (placental) and embryonic tissues (embryo proper) (Mitalipov and Wolf 2009). As development advances, the totipotent cells undergo differentiation and segregation into developmentally more restricted cell lineages. The first visual differentiation of an embryo

takes place during formation of a blastocyst, a stage consisting of two cell lineages: the inner cell mass (ICM) and the trophoblast (TE). The ICM further segregates into epiblast and primitive endoderm, which subsequently form the embryo proper and parts of the yolk sac, respectively. The trophoblast contributes to the extraembryonic tissues including primary and secondary giant cells, spongiotrophoblast and chorionic ectoderm. The epiblast and its *in vitro* counterparts, embryonic stem cells are termed pluripotent based on their ability to give rise to all three germ layers (ectoderm, mesoderm and endoderm) of the embryo proper. However, epiblast and ESCs also contribute to some extraembryonic tissues (Beddington and Robertson 1989). Early studies have demonstrated that in addition to the embryo proper, mouse ESCs contribute to the amnion and the extraembryonic mesoderm of allantois, chorion and yolk sac. Since this phenomenon has not been thoroughly investigated, it could be one of the defining factors responsible for chimera competency of ESCs. It is also important to note that developmental potential of ESCs is more restricted than that of whole ICMs (Rossant and Lis 1979). As previously stated, it is well documented that in addition to the epiblast lineage, early ICM cells also contribute to the primitive endoderm (PE) that subsequently forms part of the yolk sac. In contrast, ESCs and other pluripotent cell types are not capable of forming the PE and rely on the host embryo complementation for this extraembryonic compartment in chimeras.

The early experimental mouse chimeras were produced in the sixties by aggregating of two or more whole 8-cell embryos that resulted in normal-sized mice whose tissues consist of a mixture of parental embryos (Mintz 1962, Tarkowski 1961). Chimerism in such embryos extends to the epiblast, the trophoblast and primitive endoderm. Mouse aggregation chimeras have provided an invaluable tool to study important questions in developmental biology, cell lineage commitment, genetics and immunology (Alexandre 2001, McLaren 1976a, b, Tarkowski 1998). Gardner was able to produce mouse chimeras by an injection of isolated ICM cells into a host blastocyst cavity (Gardner 1968). Later on, as more mouse chimeric studies were conducted, it became apparent that despite introduction into the 8-cell, morula or blastocyst host embryos, the ICM contribution was always limited to the epiblast and the PE (Tam and Rossant 2003). Since the earlier studies were conducted, a range of other pluripotent cell types has been shown to contribute to mouse embryo proper chimeras. Specifically, pluripotent teratocarcinoma cells (Mintz and Illmensee 1975), ESCs (Bradley, et al. 1984), primordial germ cells (Matsui, et al. 1992), reprogrammed to the pluripotency by somatic cell nuclear transfer ESCs (ntESCs) (Wakayama, et al. 2001) and induced pluripotent stem cells (iPSCs) (Okita, et al. 2007) can also contribute to chimeras. The combination of host and donor cell types capable of forming mouse chimeras is illustrated in Fig. 1.

Mouse chimeras can be also successfully produced with tetraploid host embryos. Experimentally produced tetraploid embryos can be derived by the fusion of mouse 2-cell embryos. Such embryos are not capable of forming viable offspring; however, they have an ability to contribute to functional extraembryonic tissues. When ESCs are introduced to mouse tetraploid embryos, they colonize the embryo proper, the amnion, the allantois and the mesoderm layer of the yolk sac; while tetraploid cells are restricted to the other remaining extraembryonic tissues resulting in almost completely ESC-derived postnatal offspring (Nagy, et al. 1993). Mutant and wild type embryos have been also used for aggregation chimeras to pinpoint timing and nature of developmental defects in mutant embryos. The failure or abnormal contribution of defective embryonic cells to a particular lineage, tissue, or organ in chimeras can be complemented and rescued by the wild type embryo cells thus allowing development to continue (Tam and Rossant 2003). ESC-tetraploid chimeras can be particularly useful to study defects in development of the extraembryonic lineages. A classical example is the targeted mutation of *Hnf4*, a gene encoding for transcription factor known as Hepatocyte nuclear factor 4. Homozygous

mutant (*Hnf4^{-/-}*) embryos fail to complete gastrulation resulting in early embryonic arrest (Chen, et al. 1994). Rescue of the extraembryonic compartment in chimeras by tetraploid host embryos allowed *Hnf4^{-/-}* ESCs to undergo normal gastrulation suggesting that *Hnf4^{-/-}* defects at this stage of development are affecting primarily the PE lineage (Duncan, et al. 1997).

While conventional chimeras are generated by aggregation of embryos or embryonic cells derived from the same developmental stage, chimeric offspring can be produced by mixing embryonic cells from different developmental stages (Gearhart and Oster-Granite 1981, Nagashima, et al. 2004). As pointed above, isolated ICMs can contribute to chimeras not only when injected into the blastocoelic cavity of host blastocysts, but also when introduced into 8-cell or morula stage embryos (Butler, et al. 1987, Nagy, et al. 1990, Picard, et al. 1990, Polzin, et al. 1987, Roth, et al. 1989).

It is critical to utilize genetic, biochemical or phenotypic markers that would allow distinguishing contribution of the parental cells in a chimeric organism. Aggregation of embryos with a distinct coat color pattern (different strain of mice) has been routinely used as one of the key marker of chimerism during early research studies on chimeras. Other common markers used initially were the electrophoretic variants of the housekeeping enzyme glucose-6-phosphase isomerase (GPI). The major limitation of the GPI is the inability to detect variants at the spatial histological level (Buehr and McLaren 1981). An ideal marker would detect a putative chimera at the single-cell level in situ. The first generation of genetic markers that were used to distinguished cells of different origins in chimeras were strain-specific DNA satellite markers (Rossant, et al. 1983). These markers allowed cell lineage tracking based on in situ hybridization of histological samples. At the present time, the most broadly utilized markers are the β -galactosidase enzyme encoded by the *E. coli lacZ* gene and the green fluorescent protein (GFP). Expression of *lacZ* can be detected by histochemical staining, while GFP expression detection requires epifluorescent microscopy. Since such transgenes are not readily available for other mammal, additional genetic markers such as microsatellites, also known as short tandem repeats (STRs), can be employed (Tachibana, et al. 2012).

Chimera competent pluripotent stem cells in the mouse

As discussed, the chimera assay is used as an ultimate pluripotency test for experimental cultured mouse pluripotent stem cells, such as ESCs and more recently, iPSCs. While natural pluripotent cells within developing embryos exist transiently, when isolated and explanted using adaptive culture conditions, they can grow *in vitro* but remain pluripotent. Embryo derived stem cells termed ESCs can be propagated indefinitely, providing an unlimited source of pluripotent cells. When a few ESCs are reintroduced into a host embryo, they can resume normal developmental program and contribute to all tissues and organs of chimeric offspring. ESCs can be readily isolated using standard techniques, but only from a very few so-called “permissive” mouse strains (129, C57Bl/6 and BALB/C).

Pluripotent cell lines also have been derived from the postimplantation mouse epiblasts (day E5.5–7.5) and were termed EpiSCs (Tesar, et al. 2007). Such cells are morphologically distinct from the mouse ESCs and also differ based on their growth and culture requirements (Tesar, et al. 2007). The fundamental distinction from mouse ESCs is that EpiSCs do not contribute to chimeras (Guo, et al. 2009, Rossant 2008). Speculation is that EpiSCs represent more restricted (“primed”) pluripotent cells while ESCs symbolize developmentally more potent (“naïve”) state of pluripotency (Nichols and Smith 2009). The inability of EpiSCs to form chimeras could simply be due to their failure to contribute to the vital extraembryonic tissues. As we have discussed, the epiblast and ESCs colonize the

amion, the allantois as well as the mesoderm layer of the yolk sac. It is conceivable that their initial contribution to the extraembryonic niche is required for subsequent development into the lineages of the embryo proper.

Experimental pluripotent stem cells can be also produced by reprogramming somatic cells. Reprogrammed somatic cells may have a particularly important role in future clinical applications of autologous cell replacement therapies since the patient's own cells would evade host immune based rejection. Reprogramming of somatic cells to pluripotency can be achieved using two alternative approaches: 1) by direct reprogramming into iPSCs or 2) by somatic cell nuclear transfer into ntESCs. Direct reprogramming by current techniques operates by delivering and expressing exogenous genes known to be essential for pluripotency (Takahashi and Yamanaka 2006). Somatic cell nuclear transfer, often referred as cloning, is based on oocyte-assisted reprogramming, where the cytoplasm of an enucleated oocyte delivers the essential epigenetic and cytoplasmic factors that support natural reprogramming processes and thus recapitulates the normal development of totipotent and pluripotent cells.

Both mouse ntESCs and iPSCs share the defining feature of embryo derived ESCs; that is, their ability to contribute to chimeras (Figure 2). In addition, ntESCs and iPSCs have met the current most stringent pluripotency assays and have generated all-stem cell derived mice following tetraploid complementation (Boland, et al. 2009, Lin, et al. 2010). However, when ntESCs and iPSCs were derived from genetically identical donor cells and then compared for their ability to generate mice through tetraploid embryo complementation, only ntESCs resulted in a successful outcome; iPSCs failed to produce whole iPSC mice. This demonstrates that nuclear transfer can generate ESC-equivalent pluripotent stem cells more effectively than factor-based reprogramming (Jiang, et al. 2011).

Experimental Embryonic Chimeras in Other Species

Aggregation of early cleaving embryos (or isolated blastomeres) have resulted in the birth of chimeric animals in number of mammals including sheep (Tucker, et al. 1974), rats (Mayer and Fritz 1974), rabbits (Gardner and Munro 1974), cattle (Brem, et al. 1984) and more recently in nonhuman primates (Tachibana, et al. 2012). Moreover, aggregation of goat and sheep early embryos or injection of goat ICMs into sheep blastocysts can also result in a birth of live interspecies chimeras (Fehilly, et al. 1985, Fehilly, et al. 1984, Polzin, et al. 1987, Roth, et al. 1989). Chimeric cattle fetuses were produced by aggregation of embryos generated through somatic cell nuclear transfer with IVF embryos (Stice, et al. 1996).

In the rhesus monkey, transplantation of ICMs into blastocysts will not result in efficient integration into the host ICM (Tachibana et al 2012). Unlike mouse ICM chimeras, monkey donor and host ICMs develop into separate offspring while sharing the TE compartment of host blastocysts. Thus, whole ICM injection into a host blastocyst will often produce twin fetuses. Analysis of such rhesus fetuses demonstrated limited chimerism in their bodies (embryo proper) (Tachibana 2012). Specifically, chimerism was only detected in livers and spleens that possibly could result from the exchange of blood and blood stem cells through placental perfusions. However, chimerism in the extraembryonic compartment consisting of chorionic and amniotic tissues was extensive indicating that both the host embryo and injected ICMs contributed to these lineages.

To investigate possible mechanisms responsible for inability of injected ICMs to incorporate with host ICMs and form embryo proper chimeras, monkey blastocysts and ICMs were analyzed for lineage segregation. Cells within an ICM in mouse preimplantation stage (E3.5) blastocysts are relatively homogeneous and not visibly differentiated into epiblast and PE fates. Such segregation is apparent in peri-implantation blastocysts (E4.5), where the

layer of PE is spatially separated and covers underlying epiblast cells (Cockburn and Rossant 2010). This segregation coincides with significant decline in the ability of host ICM to incorporate injected pluripotent cells and form mouse chimeras (Ohta, et al. 2008).

Analysis of preimplantation blastocysts has demonstrated that monkey ICMs have already segregated into the epiblast and PE lineages. Even in early blastocysts, ICMs consisted of a cluster of *NANOG* positive epiblast that was covered by *GATA-6* positive PE cells. This event likely prevents efficient aggregation of injected ICMs or ESCs with donor ICMs and the formation of embryo proper chimeras.

In contrast to ICMs, monkey chimeras were efficiently produced by aggregation of cleaving 4-cell embryos (Figure 2). It was demonstrated that blastomeres of the 4-cell monkey embryos are totipotent since a single blastomere can support full-term development (Chan, et al. 2000). Between 3 to 6 individual 4-cell monkey embryos were aggregated together and 14 resulting blastocysts were transplanted into 5 recipients (Tachibana, et al. 2012). Remarkably, all 5 recipients become pregnant – 2 females carried singletons, another 2 carried twins and one recipient had quadruplets. Such high pregnancy (100%) and implantation rates (10/14 or 71%) were remarkable considering that average rhesus embryo transfer outcomes with non-chimeric embryos do not exceed 36% pregnancy and 17% implantation rates (Wolf, et al. 2004). Since chimeric blastocysts consisted of much higher cell counts (double or triple of regular numbers), it is possible that sufficient cell numbers in preimplantation embryos is critical for pregnancy initiation.

When several midgestation rhesus fetuses were recovered and analyzed for genetic contribution of parental embryos, all offspring were confirmed to be chimeras. Remarkably, chimerism was detected in all tested tissues and organs including placenta, spleen, reproductive tract, bladder, pancreas, stomach, small-intestine, adrenal gland, kidney and muscle. Since, chimeric embryos were generated by aggregating together of 3 or more cleaving embryos, some fetuses displayed contribution of multiple parental genotypes. Chimerism was also confirmed in 3 live born infants that were phenotypically normal males (Figure 2). However, detailed cytogenetic analysis of blood showed that one infant contained both male (XY) and female (XX) cells confirming gender chimerism.

ESCs in Other Species

Although derivation of ESCs or ESC-like cells expressing pluripotency markers has been reported for several species, only mouse and rat ESCs have been shown so far to contribute to germline chimeras. The first germline-competent rat ESCs were recently derived using modified culture conditions (Li, et al. 2008). These cells express typical pluripotency markers and retain the capacity to differentiate into derivatives of all three germ layers. Most importantly, they efficiently induce chimeras when reintroduced into early cleaving embryos (Li, et al. 2008). Rat ESCs were also capable of contributing to the germline and some extraembryonic lineages (Demers, et al. 2011).

It is currently assumed that species-specific differences in ESC maintenance and culture between mouse and farm animals are the posing factors challenging the identification and derivation of putative ESCs. As an example, enzymatic dissociation of cattle ESCs results in cell death or differentiation (Cibelli, et al. 1998, Stice, et al. 1996). In addition, there is inconsistency among proposed markers for defining genuine bovine ESCs (Malaver-Ortega, et al. 2012). It has been reported that livestock ESCs share some morphologic features with mouse EpiSCs rather than with mouse ESCs. These unique characteristics include flat epithelial-like cells that have low tolerance to enzymatic single cell dissociation (Tesar, et al. 2007). Several studies have reported the isolation of bovine ESC-like cells that are capable of contributing to the somatic tissues in chimeric offspring (Cibelli, et al. 1998, Saito 1992).

Putative ESCs were also described from porcine blastocysts and have been shown to contribute to some somatic tissues in chimeras (Brevini, et al. 2010, Chen, et al. 1999, Vassiliev, et al. 2010, Wheeler 1994). There was no germ line contribution of ESCs documented in these chimeras. Caprine and ovine embryo-derived cell lines have been also isolated, however pluripotency was not tested in chimeras (Behboodi, et al. 2011, Dattena, et al. 2006, Kumar De, et al. 2011, Notarianni, et al. 1991, Pawar, et al. 2009, Wells, et al. 1997).

Recent advances in iPSC technology have opened new opportunities for generation of pluripotent stem cells from many species including rat (Liao, et al. 2009), rhesus monkey (Liu, et al. 2008), cattle (Han, et al. 2011), sheep (Liu, et al. 2012) and pig (Ezashi, et al. 2009, Wu, et al. 2009). Porcine iPSCs were recently reported to induce chimeras contributing to multiple tissues that represented all 3 germ layers (West, et al. 2010). Fujishiro and co-workers demonstrated generation of naïve-like porcine iPSCs also capable of contributing to a variety of organs in chimeric fetuses (head, brachial arch, atrium, ventricle, liver, and limb bud) (Fujishiro, et al. 2012). However, no germline contribution has been observed.

Chimerism in Humans

Embryonic chimerism in humans generally occurs by spontaneous aggregation of two different zygotes or embryos. Lacking the visible features of chimerism, the condition goes underdiagnosed in most cases. Therefore, reported cases of chimerism are generally those associated with either developmental anomalies or genotype/sex discordance (Boklage 2006). One classic example of tetragametic chimerism was a woman waiting for organ donation from her biological children. However, the histocompatibility testing indicated that the children were of different genotypes. She was later confirmed to be a germ-line chimera producing two different germ cell populations (Yu, et al. 2002). Interestingly, she was phenotypically normal XX/XX chimera with no detectable chimerism in her peripheral blood.

Infertility treatments by in vitro fertilization (IVF) could potentially increase the risk of spontaneous tetragametic chimerism. Typically, improved pregnancy rates are achieved by placing more than one embryo into a patient. This can result in up to 30-to-35-fold increase in dizygotic-twin deliveries. Multiple embryo transfers could also lead to the increased risk of chimerism (Strain, et al. 1998).

Human chimerism can be also caused by aggregation of fertilized embryos with unfertilized parthenogenetic or androgenetic embryos or by aggregation with the fertilized second polar body (Malan, et al. 2006, Strain, et al. 1995).

The term microchimerism refers to a small population of donor cells in the body (fewer than 1 in 100 cells) (Gammill and Nelson 2010). Naturally acquired Mc originates primarily from fetal cells in the mother during pregnancy (fetal microchimerism) or maternal cells in her children (maternal microchimerism). Exchange of hematopoietic and other cells between twin fetuses can also result in microchimerism (De Moor, et al. 1988). Feto-maternal cell exchange starts as early as six weeks of gestation (Ariga, et al. 2001) and increases as pregnancy progresses. At 36 weeks of gestation, pregnant women have detectable fetal cells in their circulation. After delivery, the presence of fetal cells in a mother rapidly declines. However, sensitive PCR assays have shown that between 30–50% of women carry detectable fetal cells in the blood and hematopoietic tissues (e.g. spleen, lymph nodes) for several months and in some cases for decades post-partum (Evans, et al. 1999). Most of the fetal origin cells in women express CD45, the common leukocyte antigen, indicating a likely hematopoietic origin. Y-chromosome in situ hybridization techniques identified male fetal

origin cells in lung, lymph node, skin, thyroid, kidney, liver and heart of postpartum women (Koopmans, et al. 2008). The persistence of fetal cells long-term in the maternal environment strongly indicates that this population contains stem cells, termed pregnancy associated progenitor cells (PAPCs) (Khosrotehrani and Bianchi 2005). One possible origin of PAPCs is fetal hematopoietic stem cells (HSCs) as placenta has 2–4 times more HSCs than other hematopoietic tissues (e.g., liver or yolk sac) (Alvarez-Silva, et al. 2003). However, Mc is not limited to the hematopoietic lineage. For example, differentiated maternal origin cells were found among hepatocytes in liver, renal tubular cells in kidney and β -islet cells in pancreas (Oliver-Krasinski and Stoffers 2008). Male mesenchymal stem cells (presumably fetal origin) were found in bone marrow samples obtained from women who had sons from 13 to 51 years of age (O'Donoghue, et al. 2004).

The widespread presence of maternal and fetal Mc in humans in a variety of tissues and organs raises some questions about the biological significance of this phenomenon (Oliver-Krasinski and Stoffers 2008). Mouse studies suggested that fetal cells in postpartum females may be involved in tissue repair. For example, fetal cells migrated to injured liver, heart and brain tissues (Zeng, et al. 2010) (Kara, et al. 2012).

Conclusions

Naturally occurring embryonic chimeras in mammals are rare but experimental induction of chimeric animals by mixing early embryonic cells has gained a lot of momentum and is currently being used in biomedical research for potency determination of various cell types. Mouse chimeras in particular with ESCs have become a revolutionary assay to study the gene function in knockout models. The chimera assay is the most rigorous and ultimate measure of pluripotency in experimental pluripotent cells derived by the reprogramming of somatic cells. Specifically, tetraploid chimeras are considered as the most comprehensive test as it enables production of whole stem cell-derived mouse offspring. Chimerism in humans is a much more common phenomenon than originally thought. Particularly, microchimerism resulting from mutual penetration of fetal and maternal cells is common in children and their mothers.

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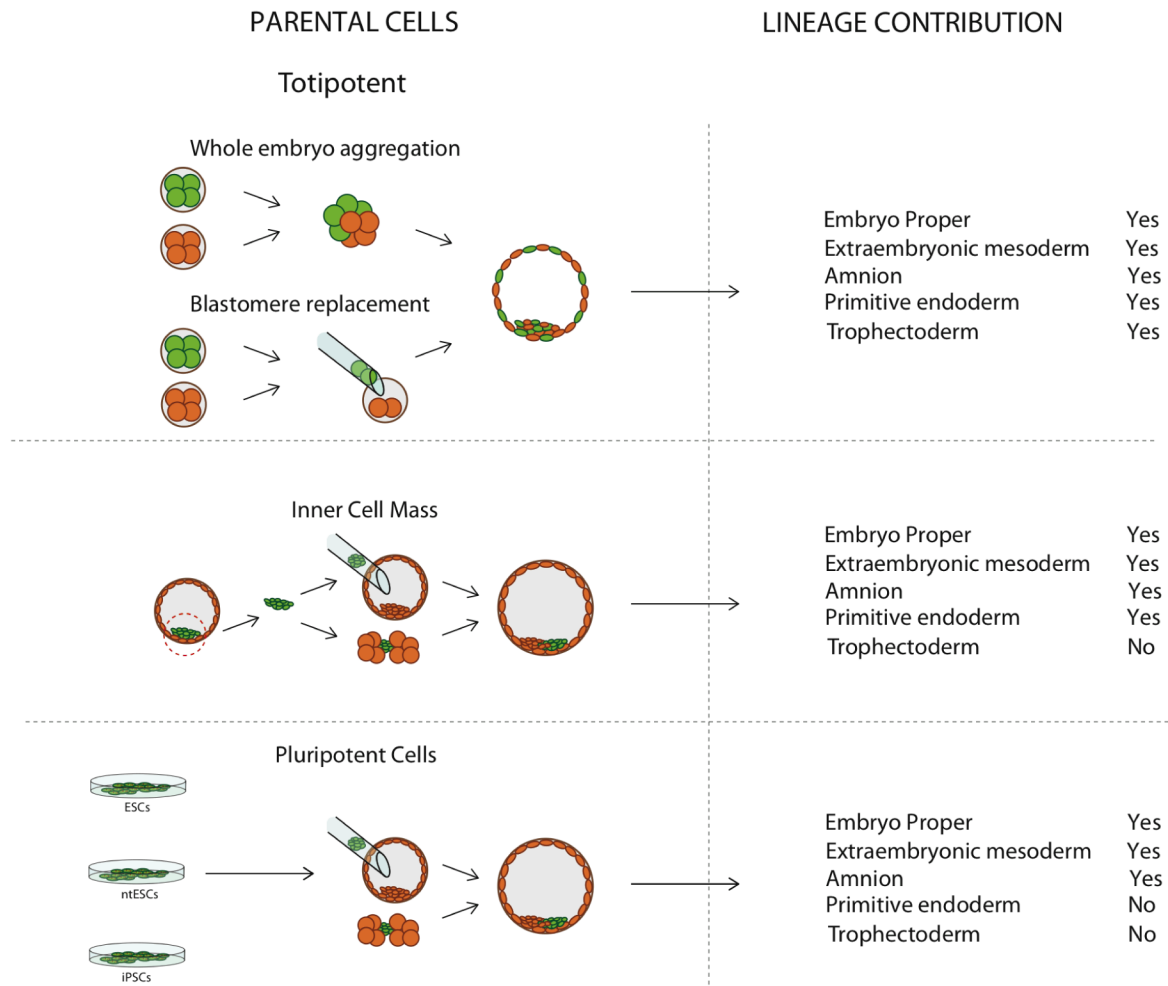


Figure 1. Developmental potential and contribution to chimeras

Aggregation of whole embryos or totipotent blastomeres from early embryos results in chimeras with contribution to all embryonic (embryo proper) and extraembryonic lineages. Whole mouse ICMs injected into blastocysts or cleaving embryos participate in the formation of the embryo proper and most extraembryonic tissues in chimeras except the trophectoderm lineage. Contribution of pluripotent ESCs, ntESCs or iPSCs in chimeras is restricted to the embryo proper, amnion and the extraembryonic mesoderm of the allantois, chorion and visceral yolk sac.



Figure 2. Mouse and rhesus monkey chimeras

Upper Panel: mouse chimeric pups produced with iPSCs in the Mitalipov laboratory (unpublished results). iPSCs were derived by transduction of skin fibroblasts isolated from C57Bl/6 (black) strain with lentiviral vectors carrying Yamanaka's reprogramming factors (Oct4, Sox2, Klf4 and c-Myc). To evaluate developmental competence, approximately 8–10 isolated iPSCs were injected into cleaving 4–8-cell embryos from ICR (albino) mice and transplanted into recipients. Note black coat color spots in offspring demonstrating contribution of iPSCs to chimeras.

Lower panel: Roku and Hex, chimeric rhesus infants each produced by aggregating of six individual 4-cell embryos (Tachibana et al., 2012). Extensive contribution of parental embryos in the blood and extraembryonic tissues was confirmed using microsatellite and mtDNA genotyping. In addition, gender specific PCR, G-banding and fluorescent in situ hybridization (FISH) based cytogenetic assays detected presence of both male (XY) and female (XX) cells in Roku suggesting sex chimerism.